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A REVIEW

Elimination of biofilm forming MRSA using phages

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Methicillin resistant *Staphylococcus aureus* (MRSA) is among the most important pathogens that forms a biofilm and thus, affecting the humans. Biofilm has always been a threat in health-care associated infections. Biofilm is formed when a micro-organism stick together on a surface and frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). This EPS is generally composed of polysaccharides, extracellular DNA and proteins. Methicillin resistant *Staphylococcus aureus* is a well known and a type of 'staph' bacteria that are resistant to many commonly used antimicrobial agents and thus, making it more difficult to treat. MRSA universally attach to surfaces and produce biofilm, especially with implants of medical devices such as urinary catheters, orthopaedic implants, cardiac pacemakers, tracheal and ventilator tubing etc. and thus, causing infections. Biofilms particularly are difficult to treat in medical situations is because of their increasing resistance to antibiotics. Therefore, there is a continuing need to develop newer methods of treating MRSA infections. Several *in-vitro* studies have shown that phages can be used to infect biofilm cells. Depolymerases can penetrate the inner layer of the biofilm by degrading the components of the biofilm exopolymeric matrix and this happens when the phages induce the production of depolymerases. The review highlights the use of bacteriophage for the control of MRSA. In addition, the concept of bacteriophage therapy may open a new horizon in controlling infections such as those caused by MRSA.

Key words : MRSA, Extra cellular polymeric substance, Bacteriophages, Depolymerase

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INTRODUCTION

Medical device related infections is an increasing burden on health-care systems with concomitant high rates of patient morbidity and mortality (Guggenbichle *et al.*, 2011). According to some reports, over 65 per cent of hospital-acquired infections occur by the infecting organisms that have the ability of producing biofilm (Del Papa *et al.*, 2007). MRSA infection causes a wide range of diseases, including endocarditis, osteomyelitis, toxic shock syndrome, pneumonia, food poisioning and carbuncles. These infections can occur in wounds or skins, burns and IV or other sites where tubes enter the body, as well as in the eyes, bones, heart or blood (Chambers, 2003). Biofilms on indwelling medical devices may be a Gram-positive or Gram-negative bacteria or yeast. The commonly Gram-positive bacteria isolated from these devices are *Enterococcus faecalis, Staphylococuss aureus,Staphylococcus epidermidis* and *Streptococcus viridians* and the Gram-negative *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis* and *Pseudomonas aeruginosa.* Biofilms may be composed of single species or multiple species, depending on the device and its duration of use in the patient, for example: Urinary catheter biofilm may initially be composed of single species, but longer exposure inevitably lead to multispecies biofilm (Stickler, 1996). Biofilm occurs step by step, such as formation of conditioning layer, bacterial adhesion, bacterial growth and biofilm expansion (Kumar, 1998) biofilm can occur on all types of surfaces such as plastic, metal, glass, soil particles, wood, medical implant materials, tissue and food products. Bacterial attachment is mediated by fimbriae, pilli, flagella and EPS that act to form a bridge between the structure (Kumar, 1998 and Deibel, 2001). Biofilm bacteria are able to tolerate significantly higher levels of antibiotics than planktonic bacteria, antibiotic resistance is reportedly upto 1000-fold greater in biofilm- bacterial cells. However, reliable methods to compare the antibiotic sensitivities of planktonic bacteria to cells in biofilms are lacking (Ceri *et al*., 1999). Phages are a kingdom of viruses that infect bacteria, and are distinct from the animal and plant viruses. Phages can have either a "lytic" or a "lysogenic" life cycle. The lytic phages are the most suitable candidates for phage therapy, because they quickly reproduce within and lyse the bacteria in their host range, growing exponentially in number in the process. Depending on the species and conditions, each "parent" phage can produce on average approximately 200 "daughters" per lytic cycle. If each daughter infects and kills a host bacterium there will be 40 000 progeny at the end of the 2nd cycle; 8 million at the end of the 3rd cycle; 1.6 billion at the end of the 4th cycle, and so on.

Phage history :

In 1896, the British bacteriologist Ernest Hankin reported antibacterial activity against *Vibrio cholerae*, which he observed in the Ganges and Jumna rivers in India. He suggested that an unidentified substance was responsible for this phenomenon and for limiting the spread of cholera epidemics. Two years later, Gamaleya, the Russian bacteriologist, observed a similar phenomenon while working with *Bacillus subtilis* (Adhya and Merril, 2006). A French-Canadian microbiologist, Felix d'Herelle, first observed in 1910 the bacteriophages phenomenon while studying microbiologic methods of controlling locusts in Mexico. In the lab, when he spread some cultures on agar, he observed round zones without growth, which he called plaques and asserted they were caused by viral parasites. Six years later, he proposed the name "bacteriophage," or bacterium-eater (Duckworth, 1976).

F. W. Twort published the first account of phages in 1915. He had noticed in his experiments that colonies of *Micrococcus* had undergone a "glassy transformation" that was transferable to other colonies (Douglas, 1975). In 1917, Felix d'Herelle independently discovered phages, he believed that "invisible microbes" were present in bacteria-free filtrates of dysentery stool samples. Emile Roux, director of the Institute, supported this endeavor and tested phages on avian typhosis and *Shigella* dysentery. Some *in vivo* studies were also performed on infected sheep, bovine hemorrhagic septicemia in Indochina, and *Pasturella multocida* in water buffalos. In addition, d'Herelle successfully treated four patients with bubonic plague using bacteriophages (Summers, 2001). The first reported phage trial was in 1921 by Bruynoghe and Maisin in France . The phages were used successfully against *Staphylococcus*; and, the overall reported phage success rate was reported at 80 to 95 per cent (Lorch, 1999 and Anonymous, 2001). In 1896, Hankins noted that existence of "anti-infectious agents" in the Ganges and Jumna rivers (Kutter, 1997 and Sulakvelidze *et al*., 2001). He did preliminary research on the effects of these agents on *Vibrio cholerae*; however, he did not continue the research further. Phages were even licensed for sale in the United States in the 1930s by such companies as Parke-Davis and Lilly (Kutter, 1997 and Sulakvelidze *et al*., 2001). Eli Lilly produced seven phage products for humans in 1940s. (Sulakvelidze *et al*., 2001). And, since 1934, phages have been successfully used. A study by the Institute reported a 90 per cent success rate against *S. aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* (Ackermann and Abedon, 2000). In addition, other eastern European countries and the former Soviet Union also had phages available on the market for years (Nelson *et al*., 2001). In 1959 - 1960, the World Health Organization (WHO) discarded phage therapy due to the success of tetracycline (Guggenbichler *et al*., 2011). On the other hand, in the 1970s, a WHO-sponsored study in Pakistan found that high dose phages seemed "equivalent to tetracycline in certain aspects of the clinical control of cholera" (Summers, 2001). In 1982, Smith and Huggins worked with phage specific to the K-1 capsule of *Escherichia coli*. They found that the phages were generally more efficient than antibiotics like streptomycin, tetracycline, ampicillin, and TMP-SMX (Barrow, 2000).

Phage biology :

Phage does not cause infection in animals. (Douglas, 1975). Phages are viruses that infect bacteria and they are the simplest and most abundantly available organisms on earth. It is said, that, there are 10 phages for each bacterial cell. Some phages are extremely specific while others have a very broad host range . As discussed earlier, phages are unable to produce disease in animals, when phages are injected into them, they will elicit the immune response for the production of antibody and this will eventually inactivate the phages (Douglas, 1975). There are at least 12 distinct groups of phages (Anonymous, 2001) and, each phage species is specific to its bacterial host. The exact morphology and genetic material (DNA or RNA) varies according to the phage species. The phage consists of a hollow head filled with phage DNA or RNA and a tunnel-like tail for injecting the genetic material into the bacteria (Skurnik *et al.*, 2007). Nevertheless, other morphological form of phage does exist. There are two types of phage, they are lytic and lysogenic. Lytic phages invade the bacterial host, rapidly replicate themselves, and then burst the host cell. In contrast, lysogenic phages integrate their genetic material into the host DNA. For phage therapy, only lytic phages are employed in order to rapidly kill the targeted micro-organism (Anonymous, 2001). For therapeutic purposes, only the lytic phages are a good choice because they cause bacterial cell lysis and do not integrate into the host DNA.

International Committee on Taxonomy of Viruses (ICTV) classify phages according to morphology and nucleic acid type (dsDNA, ssDNA, dsRNA, ssRNA). The ICTV presently recognizes one order, 13 families and 31 genera of phages. The dsDNA tailed phages, or Caudovirales, account for 96 per cent of all the phages reported in the scientific literature, and possibly make up the majority of phages on the planet (atleast 536 tailed phages are known). These phages can be grouped in three different families according to their tail type. For example: *Myroviridae* have a contractile tail whereas *Syphoviridae* has a non-contractile tail and *Podoviridae* family has no tail. There are some other methods for phage classification based on phage genome, most of which, however, categorize phages within the same group in which they are currently classified by ICTV(Ackermann and Abedon, 2000).

In humans main side effects of phages is because of the liberation of bacterial toxins as the phages lyse the bacterial cells rather than due to the bacteriophages (Kutter, 1997 and Anonymous, 2001). In addition, bacteriophages can produce effects in other aspects of the immune system. For example, it was found that patients treated with phages had lower levels of neutrophils, an increased turnover of neutrophils, and a decreased ability of these neutrophils to engulf bacteria (Weber-Dabrowska *et al.*, 2002). These effects may be due to an increased number of immature neutrophils in the circulation and that these effects were associated with a successful recovery. It has been hypothesized that this increase in the number of immature neutrophils is due to the destruction of the microbes by the phages which stimulates the release of proinflammatory cytokines that lead to neutrophil degranulation and lactoferrin release which in turn stimulate several cytokines including colony stimulating factors. These colony-stimulating factors then stimulate the release of neutrophil precursors from the bone marrow thus leading to an increase in the number of immature neutrophils (Weber-Dabrowska *et al.*, 2002). Although there is a potential for antibody production with intravenous bacteriophage treatment, bacteriophages have not been shown to have any harmful effect on human health. Thus, phage treatment has the potential to stimulate the production of neutrophils while concurrently eliminating susceptible micro-organisms.

Phage: recent experiments :

In recent years, phage therapy has been used for a variety of micro-organisms, though, rarely against *S. aureus*. Phages have been widely used to treat experimental infections. For example, the University of Florida examined *Vibrio vulnificus*-specific phages from seawater and experimented on iron-overloaded mice infected with *Vibrio*. All the untreated mice died within 24 hours of infection; while, none of the phage treated mice died (Pirisi, 2000). While, another study used phage specific to *Pseudomonas plecoglossicida* which causes bacterial hemorrhagic ascites disease in cultured ayu fish to control an experimental infection in the fish. Even more interestingly, the bacteria that became resistant to the phage became less virulent (Slopek *et al*., 1983). Also, another study used *E. coli* O157 antigen specific phages, KH1, KH4 and KH5, to control *E. coli* O157 infection *in vitro* with affecting other strains (Kudva *et al*., 1999). Another study used *Lactococcus garvieae* phages to prevent an experimental *Lactococcus garvieae* infection in yellowtail fish. Their study found that both intraperitoneal and oral administration prevented infection (Nakai *et al*., 1999). One study shows, that phages have been used to treat septic patients and thus, it was concluded that phages can be used to treat actual infections in humans (Slopek *et al*., 1983). In another study, phage treatment was administered from 1981 to 1986 to 550 cases of suppurative bacterial infections due to *Staphylococcus* and Gram negative bacteria with an improvement observed in 92.4 per cent of the patients. They found that their phages were very effective in the treatment of *S. aureus* with a sensitivity of 95 per cent (Weber-Dabrowska *et al*., 2000). A different study used phages to treat infection in cancer patients (Weber-Dabrowska *et al.*, 2000). Another study used phages to treat supperative bacterial infections (Slopek *et al*., 1983). One study developed a "serial passage" method for the isolation of long-circulating phage strains. They used the serial-passage technique to select for long-circulating *E. coli* phage lambda mutants and *Salmonella typhimurium* phage 22 mutants (Merril *et al*., 1996). This study was done to overcome the challenges to phage therapy, which is the body's immune response to phages. One study attempted use of phages to prevent infection in rabbits with an enteropathogenic strain of *E. coli* 0103 (Reynaud *et al*., 1992). Another study was more successful in using phages that attached to the K1 capsular antigen of *E. coli* to prevent septicemia and a meningitis-like infection in chickens and delayed blood bacteremia in colostrums deprived calves (Biswas *et al*., 2002). A study used a murein hydrolase from streptococcal phage C1 called lysine, which is specific for groups A, C, And E *Streptococci*. The lysine had a rapid lethal effect both *in vivo* and *in vitro* on Group A *Streptococci* (Nelson *et al*., 2001). Finally, another study successfully used phages to treat a potentially fatal vancomycin-resistant enterococcus infection in mice (Biswas *et al*., 2002).

Advantages of phage therapy :

Since phages consist mostly of nucleic acids and proteins, they are inherently nontoxic (Abedon and Thomas-Abedon, 2010 and Skurnik *et al*., 2007). However, phages can interact with immune systems, at least potentially resulting in harmful immune responses, though there is little evidence that this actually is a concern during phage treatment (Kutateladze and Adamia, 2010). Nonetheless, it can be imperative for certain phage therapy protocols to use highly purified phage preparations 8 to prevent anaphylactic responses to bacterial components, such as the endotoxins that can be found in crude phage lysates (Skurnik *et al*., 2007). Phages similarly can release bacterial components while killing bacteria *in situ*, a property associated with lysis that also can result from the application of cell-wall disrupting antibiotics. Because phages infect and kill using mechanisms that differ from those of antibiotics, specific antibiotic resistance mechanisms do not translate into mechanisms of phage resistance. Phages consequently can be readily employed to treat antibiotic-resistant infections (Carlton, 1999) such as against multi-drug-resistance *Staphylococcus aureus* (Gupta and Prasad, 2011). Phages during the bacterial-killing process are capable of increasing in number specifically where hosts are located (Carlton, 1999) though with some limitations such as dependence on relatively high bacterial densities (Abedon and Thomas-Abedon, 2010 and Capparelli *et al*., 2010). We call this auto "dosing" because the phages themselves contribute to establishing the phage dose (Abedon and Thomas-Abedon, 2010).

Challenges of phage and phage therapy :

Phages are specific to their target, the bacterial host (Summers, 2001; Sutherland *et al*., 2004 and Pirisi, 2000) , thus, the above mentioned strategies need to have a previous identification of the bacteriophages that are capable of infecting the bacterial cells and degrade the polysaccharide within the biofilm. The application of phages against biofilms may conversely be limited by two aspects: the phage specificity and secondly, the phage induced immune response. There are few studies, which report, interaction between phages and the immune system, nevertheless, it is very clear that phages are antigenic and elicit a serum antibody response, which in turn, will neutralize the phage particles. Lately, there were problems in the processing of the phages, because the laboratories were unreliable (Kutter, 1997). Titering of the phage was not done (Kutter, 1997). And after impurities have been removed, phage viability was not determined (Pirisi, 2000). Also, considerations were not given to the effects that the body would have on the phages. For example, for orally administered phages, the phages may get inactivated if the gastric acid is not reduced first (Kutter, 1997 and Anonymous, 2001).

Obstruction to phage biofilm infection :

Mostly infectious biofilm are composed of dual or multispecies, and more the number of species in a biofilm may render biofilm infection difficult. Ho (2001) has shown that planktonic cells are more rapidly lysed than biofilm cells of *Pseudomonas fluorescens*, this is because the metabolic activity of biofilm cells is reduced, especially those in the inner layer which are oxygen and nutrient bereaved. The treatment of slowly growing cells in biofilms is difficult, because phage infection and life-cycle strongly depends on the developing stage of the host bacterium (Harbarth, 2001). The biofilm matrix can act as a serious obstruction for the penetration of the phage. In fact, Doolittle *et al*.(1996) reported a partial infection of a *Pseudomonas aeruginosa* biofilm by the phage E27 in which the phage particles were not able to reach the inner layers of the biofilm. Furthermore, the biofilm matrix is also a pool of proteolytic enzymes as well as endoglucanases which can lead to bacteriophages inactivation. The dissemination limitation enforced by the biofilm matrix is obviously very reliant on the biofilm itself, which in turn is affected by the bofilm formation conditions (Simoes *et al.*, 2007). A dense biofilm structure would provide phage penetration difficult. The presence of dead cells can be considered a crisis for the progression of the infection, as they are probable viral receptor sites. Older biofilms are usually more difficult to infect, due to the increase in number of dead cells with biofilm age. Additionally, the other important property of the biofilm matrix is that, the phage action may get influenced due to its charge and hydrophobicity. Many biofilm possess an open structure with water-filled channels, which would allow the phage access to the inner biofilm layer (Sutherland *et al*., 2004).

Conclusion :

In-vitro phage experiments have shown positive results, due to the advantage that they can penetrate the inner layers of bioflms, and thus are able to infect biofilm cells and those viral particles that carry depolymerases. Several potential methods can be used to enhance phage activity. A new genes can be introduced into the phages to enable them to degrade the polymers found in the biofilm matrix. The new genes could be DNase or Proteases. Another method, a genetically engineered phage can infect a wider range of host bacterial strains and species. As a matter of choice, bacteriocins effecting elimination of the bacteria may be worth further investigation. Nevertheless, it is important to develop efficient mechanism for phage delivery. Efficient and economic phage production and its purification protocols needs to be retained before one can hope to use phage as a treatment in controlling or preventing infectious biofilms.

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